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Physiological and Histopathological Evaluation of Dithiocarbamate Toxicity to the Grass Shrimp, 'Palaemonetes Pugio'

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16. ABSTRACT

Laboratory tests were conducted to determine the acute toxicity of dithiocarbamates (Aquatreat and Busan) to molting grass shrimp and the effects of these fungicides on regenerative limb growth in grass shrimp. Results indicate that both dithiocarbamate formulations caused a dose-related inhibition or regenerative limb growth in grass shrimp. The number of days to molting was not significantly altered by Aquatreat or Busan-exposure, indicating that dithiocarbamates primarily exert their effects on the developing limb bud tissue rather than through the neuroendocrin system. Toxicity tests on Aquatreat DNM-30 (96 h LC50:127 ppb) and Busan 85 (96 h LC50: 49 ppb) revealed that dithiocarbamates are toxic to molting grass shirmp.

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PHYSIOLOGICAL AND HISTOPATHOLOGICAL EVALUATION OF DITHIOCARBAMATE TOXICITY TO THE GRASS SHRIMP, *PALAEMONETES PUGIO*

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INTRODUCTION

Dithiocarbamates are extensively used as agricultural and industrial fungicides (Melnikov, 1971). In addition, they are also applied as herbicides, plant growth regulators and nematocides. Because of their widespread agricultural usages, dithiocarbamates are likely to enter the aquatic environment through runoff into rivers and estuaries. Nevertheless, with few exceptions (Davis, 1961; Tooby, Hursey, and Alabaster, 1975; Lindén et al., 1979; Bowman, Oller, and Cairns, 1981), little was done to assess the toxic effects of dithiocarbamates on aquatic organisms. Consequently, studies were initiated to assess the toxic and sublethal effects of two dithiocarbamate formulations; Aquatreat^{R} DNM-30 (active ingredients, sodium dimethyldithiocarbamate and disodium ethylene bisdithiocarbamate) and Busan^{R} 85 (active ingredient, potassium dimethyldithiocarbamate) on the estuarine grass shrimp, Palaemonetes pugio.

Since earlier investigations in this laboratory (Conklin and Rao, 1978; Rao et al., 1981), as well as studies by other investigators (Lee and Buikema, 1979; Price and Uglow, 1979; Saroglia and Scarano, 1979), have shown that crustaceans tend to exhibit increased sensitivity to aquatic pollutants in relation to molting (ecdysis), tests were conducted to determine the acute toxicty of Aquatreat and Busan to molting grass shrimp. In addition, since crustacean limb regeneration has been shown to be a useful sublethal bioassay to test the effects of pentachlorophenol (Rao, Conklin, and Brannon, 1978) and other chlorophenols (Rao et al., 1981), studies were conducted to assess the effects of dithiocarbamates (Aquatreat and Busan) on regenerative limb growth in grass shrimp.

During the course of toxicity and limb regeneration studies, it was observed that a number of shrimp exposed to the dithiocarbamates developed

darkened gills. In view of this observation, and the fact that dithiocarbamates have been noted to cause pathological responses in developing chick embryos (Van Steenis and Logten, 1971), developing Xenopus larvae (Bancroft and Prahlad, 1976; Prahlad, Bancroft, and Hahzely, 1974; Anderson and Prahlad, 1976) and in thyroids of rats (Ivanova-Chemichanska, Markov, and Dashev, 1971), histopathological and ultrastructural studies were done to evaluate the nature of the gill blackening response. Subsequently, in view of the noted response of gills to dithiocarbamate exposure, studies were also conducted to determine the effects of Aquatreat and Busan on respiration in grass shrimp.

MATERIALS AND METHODS

Grass Shrimp

Adult grass shrimp (Palaemonetes pugio) were collected from grass beds located in Santa Rosa Sound, Gulf Breeze, Florida. Following collection, the shrimp were held in 84.5 liter glass aquaria containing aerated, recirculating seawater and an oyster shell biological filter. The shrimp were fed a daily diet of freshly hatched brine shrimp (Artemia) supplemented with Tetramin^{R} staple fish food flakes. Grass shrimp were acclimated to the individual test conditions for a minimum of 96 hr prior to usage in experiments, and all studies were conducted with nongravid shrimp in known stages of the molt cycle. The molt stage of individual shrimp was determined prior to each experiment by microscopic examination of the outer uropod edges for evidence of apolysis (epidermal retraction) and formation of new setae (for further details, see Conklin and Rao, 1978).

General Experimental Conditions

Grass shrimp were exposed individually in glass jars (8.5 cm dia. x 9.5 cm high) that contained 250 ml of test medium, or seawater alone in the case of the controls. Although the solutions were not aerated, they were

renewed every 24 hr in order to provide fresh oxygenated seawater and toxicant as well as to remove accumulated waste products. The dissolved oxygen content and pH of the solutions were determined initially and just prior to changing solutions. The salinity (10 °/oo), temperature (20 ± 1°C) and pH (7.8-8.0) were the same for all experiments. A controlled incubator was used to maintain the required temperature, while a 12 hr light--12 hr dark photoperiod was provided by cool-white fluorescent illumination. The low salinity seawater was obtained by diluting natural seawater (5 µm filtered) with distilled water. Feeding was discontinued during 96 hr tests; however, for exposures longer than 96 hr the animals were fed live brine shrimp (Artemia) nauplii on alternate days.

Dithiocarbamates

The two dithiocarbamate formulations tested were Aquatreat^{R} DNM-30 (registered trademark, Alco Chemical Corp., Chattanooga, TN) and Busan^{R} 85 (registered trademark, Buckman Laboratories, Memphis, TN). The active ingredients in Aquatreat DNM-30 are sodium dimethyldithiocarbamate (15%) and disodium ethylene bisdithiocarbamate (15%), while the active ingredient in Busan 85 is potassium dimethyldithiocarbamate (50%). Since both of these products are aqueous solutions, the test stock solutions were prepared by volumetric dilution of the carbamates with deionized distilled water. Suitable amounts of the stock solutions were added to filtered (5 µm) seawater (10 °/oo) salinity) to obtain the desired test concentration. All test concentrations reported are nominal concentrations based on the calculated amount (v/v) of the total product present.

Acute Toxicity Tests

Very late premolt (stages D_2-D_4) grass shrimp, all of which were within 48 hr of ecdysis (molting), were exposed to 75, 100, 125, 150 and 175 ppb (nℓ/ℓ)

Aquatreat and 10, 20, 40, 60 and 80 ppb Busan for 96 hr; these concentrations were determined from range-finder tests. A minimum of 20 shrimp were exposed at each test concentration and also as seawater controls. At 24 hr intervals, prior to changing solutions, the test jars were examined for cast exuvia (evidence of molting) and for shrimp mortalities.

Limb Regeneration

Experiments were conducted to determine the effects of Aquatreat and Busan on limb regeneration in grass shrimp. Groups of 24 shrimp each were exposed to each of three concentrations of either Aquatreat or Busan, while two additional groups of 24 shrimp each served as seawater controls. The exposure concentrations were established such that the highest concentration tested was lower than the LC_{10} value computed from the acute toxicity tests.

The experimental methods used to study limb regeneration were similar to those described earlier (Rao, Conklin, and Brannon, 1978). Since adequate time prior to ecdysis is required for successful limb bud formation, regeneration tests were initiated utilizing only intermolt shrimp (stage C). Immediately following removal of the left fifth pereiopod, the shrimp were randomly placed in either experimental or control media. On the 4th, 7th, 9th and 11th day after limb removal the shrimp were examined microscopically to assess limb bud growth and progress in the molt cycle. In order to compare regenerative growth for different shrimp, a regenerative index (R-value) was calculated according to the formula of Bliss (1956):

$$\text{R-value} = \frac{\text{length of limb bud}}{\text{length of carapace}} \times 100$$

The R-values from any shrimp which molted prior to day 12 were not included in the computation of the average R-values.

Respiration Studies

In this series of experiments, groups of ten grass shrimp (stage C initially) each were exposed to Aquatreat (60, 80 and 100 ppb) and Busan

(5, 10, and 20 ppb) for up to 11 days to assess the effects of dithiocarbamates on oxygen consumption. The shrimp were fed on a schedule that allowed for a minimum 24 hr interval between feeding and respirometry. Measurements of oxygen consumption were made using a Gilson Differential Respirometer. Respirometry readings were taken every 15 minutes over a 90 minute period on days 0, 2, 4, 7, 9, and 11. Oxygen consumption was measured at the exposure temperature ($20 \pm 1^{o}C$), and at approximately the same time each day. Excess moisture was blotted from each shrimp and then the wet weights were determined on a Mettler H54AR balance. The oxygen consumption data are expressed as $\mu\ell$ O_2 consumed/g wet weight/hr.

Analysis of Results

Data analysis was performed using the Statistical Analysis System (SAS) Computer package (Barr et al., 1976). The 96 hr LC_{50}s and their corresponding 95% fiducial limits were computed from the acute toxicity data using the SAS Probit Procedure, while the regeneration and respiration data were analyzed using the SAS General Linear Model (GLM) procedure and Duncan's Multiple Range Test to test for significant (P = 0.05 level) effects within treatments. The EC_{50}s for inhibition of limb bud growth were computed using the probit procedure.

Histological and Ultrastructural Procedures

A series of experiments were conducted to assess the effects of dithiocarbamates on grass shrimp at the cellular and subcellular levels. In experiment 1, five groups of 15 shrimp each were exposed to 5-60 ppb Busan in 10 °/oo seawater for 14 to 17 days. In experiment 2, five groups of shrimp were exposed to 40-140 ppb Aquatreat in 10 °/oo seawater for 96 to 120 hr; while in a third experiment, five groups of 20 shrimp each were exposed to 40-120 ppb Aquatreat in 10 °/oo seawater for 28 to 35 days. Following exposure, the shrimp were sacrificed, and the gills were dissected

out and processed for histological and ultrastructural examination using previously described methods (Doughtie and Rao, 1978, 1981; Bell et al., 1969).

RESULTS

Acute Toxicity

All of the late premolt shrimp (stages D_2-D_4) employed in these tests have undergone ecdysis during the initial 48 hr of the 96 hr exposure period. Although all of the control shrimp molted successfully and survived the remainder of the test, Aquatreat and Busan-exposed shrimp exhibited dose-related mortalities. The LC_{50} values and the respective 95% fiducial limits are shown in Table 1. Busan 85 which has a higher percentage of active ingredient was the more toxic of the two biocides tested.

Effects of Aquatreat DNM-30 and Busan 85 on Limb Regeneration

Aquatreat-exposed grass shrimp (Fig. 1) and Busan-exposed shrimp (Fig. 2) exhibited a marked dose-related inhibition of regenerative limb bud growth as compared with control shrimp. Examination of shrimp on the 4th day following limb removal revealed that all control shrimp had successfully completed wound healing, and had initiated basal limb bud growth; however, carbamate-exposed shrimp exhibited little or no basal limb growth. Statistical analyses utilizing the SAS/GLM Procedure followed by Duncan's Multiple Range Test showed that for day 4 the R-values for all Aquatreat concentrations tested (40, 60 and 80 ppb) were significantly ($P < 0.05$) less than those of the controls; for days 7,9 and 11, the R-values for the 60 and 80 ppb Aquatreat-exposed shrimp were significantly ($P < 0.05$) lower than the control values. In the Busan 85 regeneration experiment, the day 4, 7, 9 and 11 R-values for the 5 and 10 ppb Busan-exposed shrimp were significantly ($P < 0.05$) less than those of the controls. The computed EC_{50}s for inhibition of regenerative limb growth by Aquatreat and Busan were 43 ppb (39-47 ppb) and 3.8 ppb (2.9-4.5 ppb) respectively based on day-4 R-values.

Although the dithiocarbamates tested significantly inhibited regenerative limb growth, the number of days to molting was not significantly ($P > 0.05$) altered by either Aquatreat or Busan exposure. The number of days to molting for 40,60 and 80 ppb Aquatreat-exposed shrimp and the seawater control shrimp were 16.14 ± 0.78, 14.35 ± 0.93, 15.00 ± 0.98 and 15.82 ± 1.10 (mean ± S.E.) days respectively. For 2.5, 5 and 10 ppb Busan-exposed shrimp and the seawater control shrimp, the number of days to molting were 14.21 ± 0.64, 13.83 ± 0.59, 14.15 ± 0.69 and 14.42 ± 0.59 (mean ± S.E.) days respectively.

Effects of Aquatreat DNM-30 and Busan 85 on Respiration in Grass Shrimp

Respiration measurements for Aquatreat and Busan exposed shrimp indicate that these two dithiocarbamate formulations inhibit the rate of oxygen consumption by grass shrimp. During the course of the respiration experiments, the control oxygen consumption rates ($\mu\ell$ O_2/g wet weight/hr) exhibited a gradual increase concurrent with progress in the molt cycle. However, for Aquatreat-exposed shrimp (Fig. 3) and Busan-exposed shrimp (Fig. 4) the average rates of oxygen consumption declined relative to the control rates. After 7, 9 or 11 days exposure to 80 and 100 ppb Aquatreat and 4, 7, 9 or 11 days exposure to 10 and 20 ppb Busan, the observed respiration rates were significantly (Duncan's Test; $P < 0.05$) less than the control values.

Histopathology of Gills

a. Effects of Busan-85

The fine structural changes occurring in the gill axes and lamellae of shrimp exposed to Busan (experiment 1) have recently been described in detail (Doughtie and Rao, 1982); for the purpose of comparison, a brief summary of these observations follows. In contrast to the normally translucent nature of the pleurobranchiae (Fig. 5) and the regular arrangement of long, narrow, smoothly profiled lamellar plates on the gill axes (Fig. 6) (see also Doughtie and Rao, 1978, 1982), the gills of 21 out of 39 shrimp which survived

the two week exposure to 5.0-60.0 ppb Busan appeared grossly "milky" or brownish-black (fig. 7). Primary degenerative changes were noted in the lamellar apices of "milky" gills and included: swollen mitochondria, dilated rough endoplasmic reticulum, a loss of microvilliform elaboration of the subcuticular epithelial cell plasmalemma, the formation of autophagosomes, the appearance of an unusual (fine-grained, moderately-dense) material in the subcuticular space, and the occurrence of paracrystalline structures in the cytoplasm of epithelial cells as well as in the axis podocytes. Secondary changes included: the phagocytosis and encapsulation of degenerating lamellar epithelial and glandular cells by granular hemocytes, and an increase in mitotic activity by cells in the basal lamellar regions. In shrimp possessing brownish-black gills, the discoloration was invariably caused by the inclusion of dense, presumably melanized, hemocyte plugs (up to 0.5 mm in length) that replaced the apical lamellar tissues (fig. 8). In premolt shrimp with hemocyte plugs, a new but abnormally thickened and contorted cuticle was formed subjacent to the plug. The occassional, premature death of one or more cells responsible for forming the new cuticle appeared to cause perforations on the latter. At ecdysis the hemocyte plugs were sloughed resulting in markedly truncated lamellae with amorphic apical scabs (fig. 9). In 6 of the 13 shrimp which survived the 2 week exposure to the higher concentrations (20.0-60.0 ppb) entire pleurobranchiate gill processes were sloughed; and in one surviving shrimp, only 3 of the original 10 pleurobranchiae persisted.

b. Effects of Aquatreat DNM-30

Eight animals that were exposed to 60-140 ppb Aquatreat for 96-120 hrs. (experiment 2), and which had developed black-gills, were examined ultrastructurally. Various degrees of lamellar degeneration, resulting in both "milky" and black-gills, were observed not only between the lamellae of individual shrimp, but also between the gills of different shrimp. Many of

the observed degenerative features (fig. 10) were similar to those which characterized the "milky" and blackened lamellae of shrimp exposed to Busan for 14 days. These similarities, included in the order of their presumed severity are as follows: 1) an increased number of epithelial cell lysosomes and autophagasomes as well as a reduction in the degree of subcuticular microvilliform elaboration of the epithelial cell plasmalemma; 2) the occurrence, in the subcuticular spaces of some lamellae, of a dense, grainy material of uncertain origin, and 3) the involvement of granular hemocytes in the phagocytosis of degenerate lamellar epithelium; and the accumulation of hemocytes in the lamellar apices to form a presumably melanized plug, responsible for the brownish-black lamellar discoloration. In contrast to these similarities, there were a number of differences between the branchial degenerative responses of shrimp exposed to Aquatreat and Busan. Perhaps most notable of these differences was the abnormally electron-dense appearance of the branchial hemolymph in half of the Aquatreat-exposed shrimp examined (figs. 10,11,12). In these animals, large lipid-like droplets with an unusual matrical substructure were frequently observed adjacent to or within the hemolymph channels (figs. 12,13). In shrimp with extensively blackened gills, the axis podocytes exhibited a marked deterioration in the pedicellar elaboration of the plasmalemma such as was also observed in the podocytes of some shrimp exposed to Busan. A distinguishing feature, however, was the abnormally electron-dense nature of the cytoplasm and central vacuole fluids in the podocytes of Aquatreat-exposed shrimp (figs. 14,15).

In shrimp examined after exposure to 60-120 ppb Aquatreat for 28-35 days, ultrastructural abnormalities were noted primarily in lamellar mitochondria; the severity of the abnormalities, however, was not dependent on the concentration of Aquatreat in the exposure media. The ultrastructural changes were first detected as small, polymorphic, moderately-dense

inclusions within the matrices of slightly swollen mitochondria (fig. 16). In other shrimp, the mitochondria exhibited a greater degree of swelling but the dense inclusions were not as conspicuous (fig. 17). Finally, in two of the examined shrimp, the lamellar mitochondria possessed characteristics of megamitochondria (fig. 18). The morphological events which precipitated this presumed sequence of changes appear to have occurred in the following order. Concurrent with the dispersion of the matrical granules, amorphous patches of moderately lucent, lipid-like material appeared in the mitochondrial matrices (fig. 19); associated with this change was the presence, in the subcuticular epithelial cytoplasm, of numerous particulate congregations (particle size, .007-0.01mm diameter). These moderately dense congregations appeared to condense to form smaller, somewhat spherical, electron-dense cytoplasmic inclusions (fig. 20). Frequently, the particulate inclusions were in close apposition to the outer membranes of slightly swollen mitochondria which were subjacent to the lamellar cuticle (fig. 21). It was in the matrices of many of these, and other comparably swollen mitochondria, that the extremely electron-dense inclusions (fig. 22) occurred. These inclusions appeared to arise from the coalescence of a flocculent material that was also associated with the outer surfaces of the cristae (fig. 23). It is of interest to note that a similar dense flocculent material and associated inclusions were frequently observed in the central vacuole of axis podocytes in shrimp with lamellar mitochondrial inclusions (figs. 24,25). The development of lamellar megamitochondria in the two shrimp was associated with mitochondria that possessed matrical inclusions and appeared to be the result of abnormal mitochondrial fusion (fig. 26).

The only other abnormality found in the gills of shrimp subjected to long-term exposure to Aquatreat was the dilation of the lamellar plates in which megamitochondria were found (fig. 18). In contrast to the gills of

shrimp exposed to Aquatreat for 3-4 days or Busan for 14 days, widespread cellular degeneration and phagocytic uptake by large numbers of granular hemocytes was not noted in gills of shrimp subjected to a long-term exposure to Aquatreat.

DISCUSSION

Comparative Toxicity of Dithiocarbamates

The 96-hr LC_{50} values obtained for Aquatreat DNM-30 (127 ppb) and Busan 85 (49 ppb) in tests with molting grass shrimp are considerably lower than the previously reported dithiocarbamate LC_{50} values for fishes. For example, Tooby, Hursey, and Alabaster (1975) reported 96-hr LC_{50} values of 0.53 to 250 ppm (530 to 250,000 ppb) for several different dithiocarbamate fungicides in tests with the harlequin fish (Rasboro heteromorpha). They observed marked differences in toxicity for different salts of the same dithiocarbamate. The LC_{50} for 70% Zineb (zinc ethylene bisdithiocarbamate) was 250 ppm, while for 93% Nabam (disodium ethylene bisdithiocarbamate) and 80% Maneb (manganese ethylene bisdithiocarbamate) the LC_{50}s were 0.7 ppm and 0.53 ppm respectively. Linden et al. (1979) reported a 96-hr LC_{50} of 0.52 ppm for 85% Maneb using the brackish water fish Alburnus alburnus; however, in tests on the same compound with the copepod, Nitocra spinipes a lower 96-hr LC_{50} (0.11 ppm) was obtained. In other tests with Maneb (98 + %), the 18-hr LC_{50} for the cladoceran crustacean, Daphnia pulex, has been reported to be 96 ppb (Bowman, Oller, and Cairns, 1981). Thus, it appears that crustaceans may be more sensitive to dithiocarbamate exposure than are fish.

Effects of Aquatreat DNM-30 and Busan 85 on Limb Regeneration

Exposure to dithiocarbamates caused dose-related inhibition of regenerative limb growth in grass shrimp. The 96-hr EC_{50}s for inhibition of regenerative limb growth by Aquatreat (43 ppb) and Busan (3.8 ppb) are lower than

the 96-hr LC_{50}s (127 ppb and 49 ppb, respectively) from toxicity tests using molting grass shrimp. As observed in studies with sodium pentachlorophenate (Rao, Conklin, and Brannon, 1978) and other chlorophenols (Rao et al., 1981), the dithiocarbamates inhibited limb regeneration without concurrently altering the duration of time to molting. These results are in contrast to the reported effects of certain other pollutants on crustacean limb regeneration. For instance, in tests with fiddler crabs, DDT accelerated the rate of limb regeneration and shortened the time to molting (Weis and Mantel, 1976), while heavy metals (Weis, 1976, 1977; Weis and Weis, 1979) and polychlorinated biphenyls (Fingerman and Fingerman, 1979, 1980) inhibited regenerative limb growth lengthened the time to molting. These chemicals (DDT, heavy metals, and PCBs) seem to exert their effects by acting primarily on the neuroendocrine system, which controls molting and related processes; in contrast, dithiocarbamates and chlorophenols seem to affect regeneration by acting directly on the developing limb bud tissues. Previous studies showed that dithiocarbamates can affect development in chick embryos (Van Steenis and Logten, 1971), amphibian (Xenopus) larvae (Bancroft and Prahlad, 1976), and regenerating forelimbs in the newt Triturus cristatus (Arias and Zavanella, 1979).

Effects of Aquatreat DNM-30 and Busan 85 on Respiration in Grass Shrimp

In view of the observation that a number of Aquatreat/Busan-exposed grass shrimp developed darkened gills, it was not surprising that the rates of oxygen consumption were inhibited by exposure to the carbamates. In earlier studies (Cantelmo et al., 1978) involving exposure to sodium pentachlorophenate, the oxygen consumption rates of exposed shrimp (stage C) initially increased somewhat and then declined rapidly. Similar to 2,4-dinitrophenol, sodium pentachlorophenate has been shown to be an uncoupler of oxidative phosphorylation (Garbus and Weinbach, 1963). Although the specific biochemical mode of action for dithiocarbamate toxicity is incompletely understood,

dithiocarbamates are known to be inhibitory to metal-dependent enzymes and sulfhydryl containing enzymes (Schlagbauer and Schlagbauer, 1972). It is believed that the effects of the salts of dithiocarbamic acids on microorganisms are based on disruption of the oxidative-reductive processes (Melnikov, 1971).

Gill Blackening in Grass Shrimp

The occurrence of black gills in grass shrimp subjected to relatively long-term exposure to Busan or short-term exposure to Aquatreat is not unique. The black gill response has been reported previously for shrimp exposed to cadmium (Nimmo, Lightner, and Bahner, 1977; Couch, 1977) and in shrimp subjected to protozoan (Couch, 1978) or fungal infections (Nimmo, Lightner and Bahner, 1977; Bian and Egusa, 1981). The black-brown discoloration which occurs in the traumatized gills of crustaceans has largely been attributed to the production of melanin by hemocytes responding to the impacted regions of the gills (e.g. Unestam and Nylund, 1972; Lightner and Redman, 1977). Recently, we presented a detailed description of the ultrastructural changes leading to black gill development in shrimp exposed to Busan-85 (Doughtie and Rao, 1982) and emphasized the similarities between these changes and those changes which occur during crustacean wound healing and regeneration. The results of the latter study and this study seem to indicate that the dithiocarbamate formulations (Busan and Aquatreat) exert a toxic influence directly upon the lamellar epithelium. Furthermore, the phenomenon of black-gill development in shrimp exposed to dithiocarbamates appears to be mediated by granular hemocytes which phagocytose and encapsulate degenerate epitheluim so as to isolate these damaged tissues from viable basal lamellar tissues prior to ecdysis (see Doughtie and Rao, 1982). This hypothesis differs from one proposed by Nimmo, Lightner, and Bahner (1977) in which it was suggested that hemocyte accumulations in the lamellar apices of shrimp exposed to cadmium were due to the migration of toxicant-laden hemocytes from other

regions of the body to the gills where they would be sloughed.

Some of the primary ultrastructural changes reported here and in a previous paper (Doughtie and Rao, 1982) for the gills of shrimp exposed to dithiocarbamates seem to reflect the disruptive or inhibiting effect that dithiocarbamates are known to have on oxidation-reduction processes (Melnikov, 1971; Ivanova-Chemichanska, Markov, and Dashev, 1971; Briquet, Sabadie-Pialoux, and Goffeau, 1976), glucose metabolism (Dailey, Leavens, and Walton, 1969), and on protein synthesis (Ivanova-Chemichanska, Markov, and Dashev, 1971; Melnikov, 1971). In fact, the low amplitude mitochondrial swelling and dilation of rough endoplasmic reticulum as observed in the gills of shrimp exposed to Busan-85 for 14 days (experiment 1), (see Doughtie and Rao, 1982) are similar to the changes observed in the thyroids of rats following ethylene bis-dithiocarbamate administration (Ivanova-Chemichanska, Markov, and Dashev, 1971). It is interesting to note, however, that the development of mitochondrial inclusions and megamitochondria was widespread only in the gills of shrimp subjected to long-term exposure to Aquatreat(experiment 3). The dense matrical inclusions reported here are very similar to the mitochondrial inclusions of vertebrate steriod producing cells (e.g. Sheridan and Belt, 1964) and kidney tubule cells (Yamamoto, Ebe, and Kobayashi, 1969). In the latter cells, the inclusions are thought to be lipid in nature and reflect unusual metabolic activity. The reason(s) for the subsequent development of megamitochondria is not clear, although it has been proposed that megamitochondria formation in yeast cells exposed to copper resulted from the hyperproduction of cytochromes (Keyhani, 1973). Megamitochondria were also reported in the gills of grass shrimp exposed to sodium pentachlorophenate (Na-PCP) (Doughtie and Rao, 1978).

SUMMARY

Toxicity tests on Aquatreat DNM-30 (96-hr LC_{50}: 127 ppb) and Busan 85 (96-hr LC_{50}: 49 ppb) revealed that dithiocarbamates are highly toxic to molting grass shrimp (Palaemonetes pugio). The results of these tests, when coupled with other reports on dithiocarbamate toxicity, suggest that crustaceans are more sensitive to dithiocarbamates than are fish. In limb regeneration studies, both of the dithiocarbamate formulations caused a dose-related inhibition of regenerative limb growth in grass shrimp; the day-4 (96-hr) EC_{50}s for inhibition of regenerative limb growth were lower than the 96-hr LC_{50} values. Since the number of days to molting was not significantly altered by Aquatreat or Busan-exposure, dithiocarbamates appear to primarily exert their effect directly on the developing limb bud tissue rather than through the neuroendocrine system.

During the course of exposure to the dithiocarbamates, some of the shrimp developed gross abnormalities in gill morphology. Respiration studies revealed that Aquatreat and Busan-exposed grass shrimp exhibited lower rates of oxygen consumption than did control shrimp. Histological and ultrastructural examinations revealed a gradation of pathological responses in the carbamate-exposed gills--including a massive influx of hemocytes into degenerating gill lamellae, the formation of presumably melanized plugs into lamellar apices, and the sloughing of these plugs and surrounding cuticle at ecdysis--leading to lamellar truncation and to degeneration of entire pleurobranchial gill processes. The observed defensive response of granular hemocytes to dithiocarbamates is comparable to some of the previously described wound healing responses in ocher crustaceans.

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Table 1. Toxicity of Aquatreat DNM-30 and Busan 85 to molting grass shrimp, Palaemonetes pugio (from Conklin and Rao, 1982).

Toxicity value	Aquatreat DNM-30[a]	Busan 85[a]
96-hr LC_{10} (95% f.l.)	86 (54-101)	17 (3-26)
96-hr LC_{50} (95% f.l.)	127 (113-145)	49 (42-57)
96-hr LC_{90} (95% f.l.)	169 (149-212)	80 (70-100)

[a] concentrations in ppb by volume (nℓ/ℓ)

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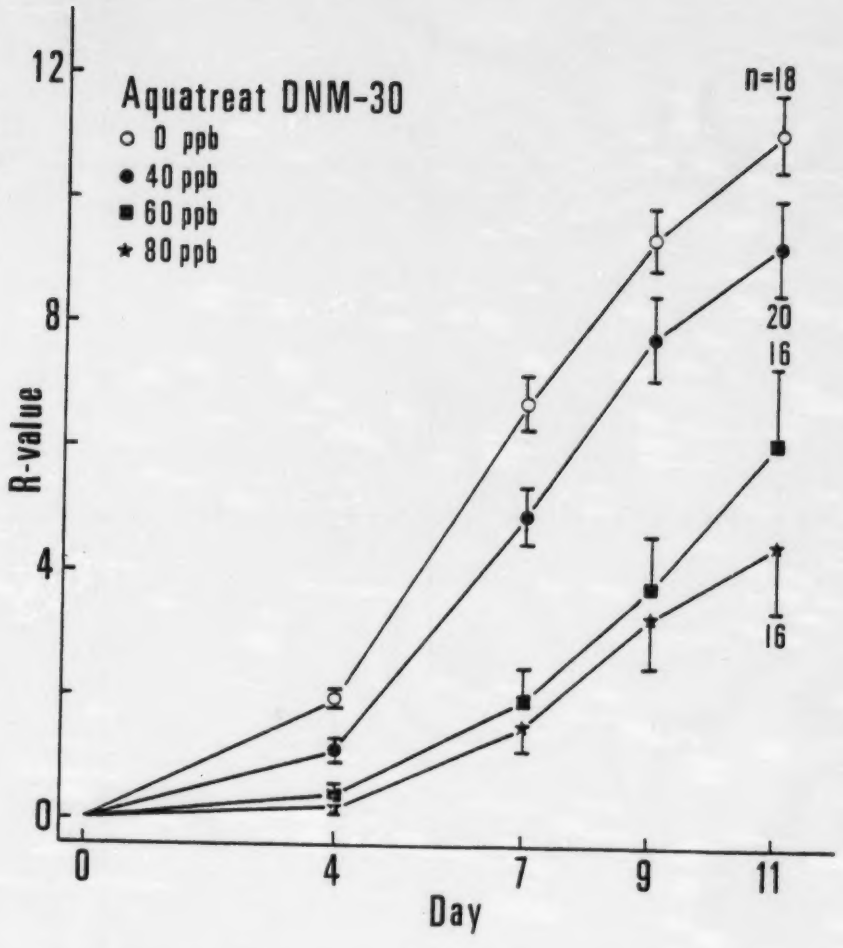

Fig. 1 Effect of Aquatreat DNM-30 on regeneration of the fifth pereiopod of the grass shrimp, Palaemonetes pugio. Each data point shows the mean regenerative index (R-values) for a given day, while the vertical lines represent the standard error of the mean. The numbers of shrimp (n) for day 4,7, and 9 were the same as those given for day 11. All concentrations are in ppb (nℓ/ℓ) of the formulation. Figure is redrawn from data of Conklin and Rao (1982).

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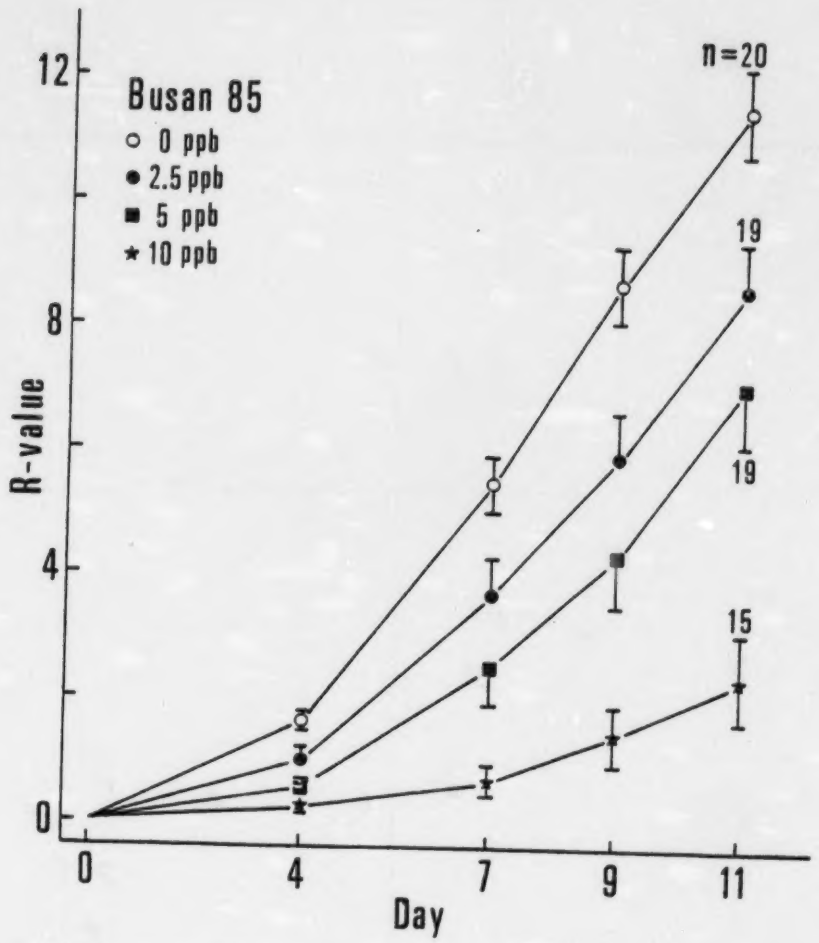

Fig. 2 Effect of Busan 85 on regeneration of the fifth pereiopod of the grass shrimp, Palaemonetes pugio. Each data point shows the mean regenerative index (R-values) for a given day, while the vertical lines represent the standard error of the mean. The numbers of shrimp (n) for days 4,7, and 9 were the same as those given for day 11. All concentrations are in ppb (nℓ/ℓ) of the formulation. Figure is redrawn from data of Conklin and Rao (1982).

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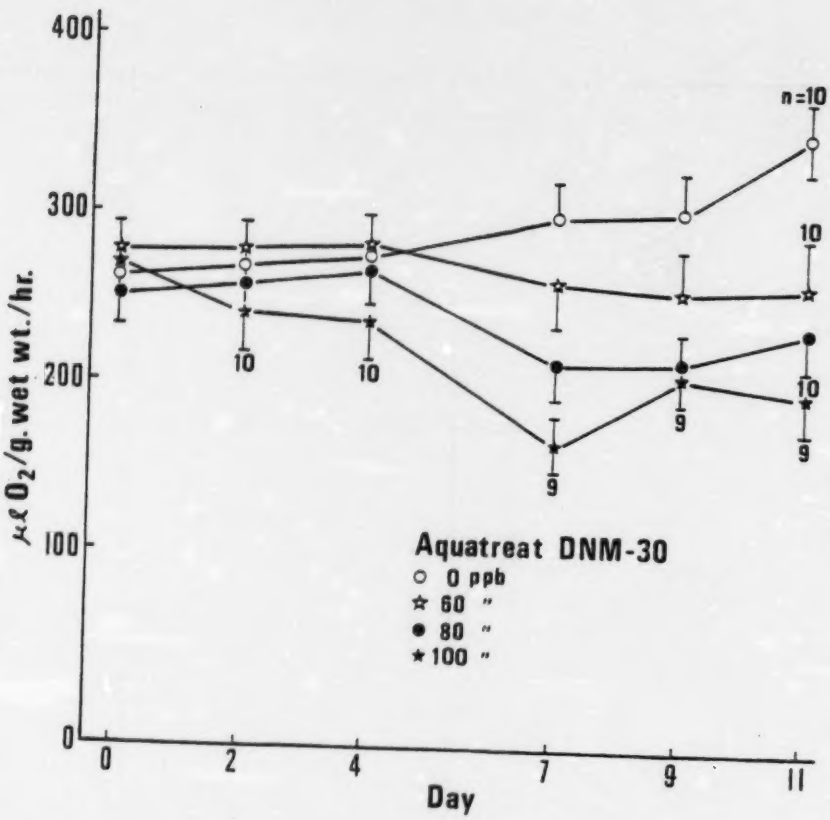

Fig. 3 Effect of Aquatreat DNM-30 on oxygen consumption in the grass shrimp, Palaemonetes pugio. Each data point shows the mean ± the standard error of the mean. The number of shrimp (n) shown for a given concentration was the same for all days except as noted. All concentrations are in ppb (nℓ/ℓ) of the formulation.

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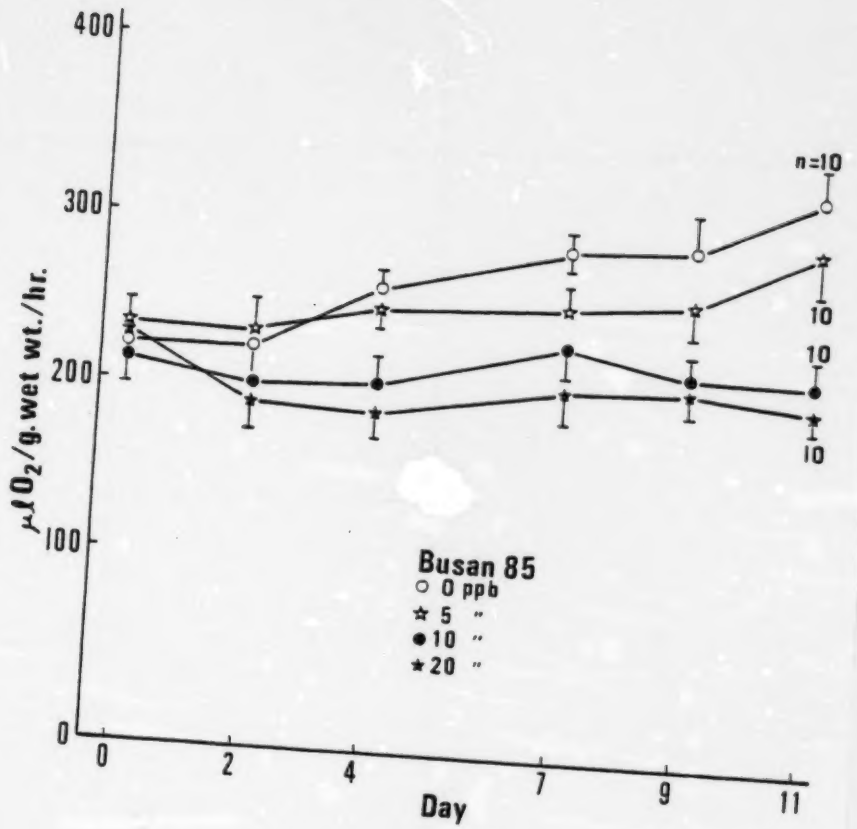

Fig. 4 Effects of Busan 85 on oxygen consumption in the grass shrimp, Palaemonetes pugio. Each data point shows the mean ± the standard error of the mean. The number of shrimp (n) shown for a given concentration was the same for all days. All concentrations are in ppb (nℓ/ℓ) of the formulation.

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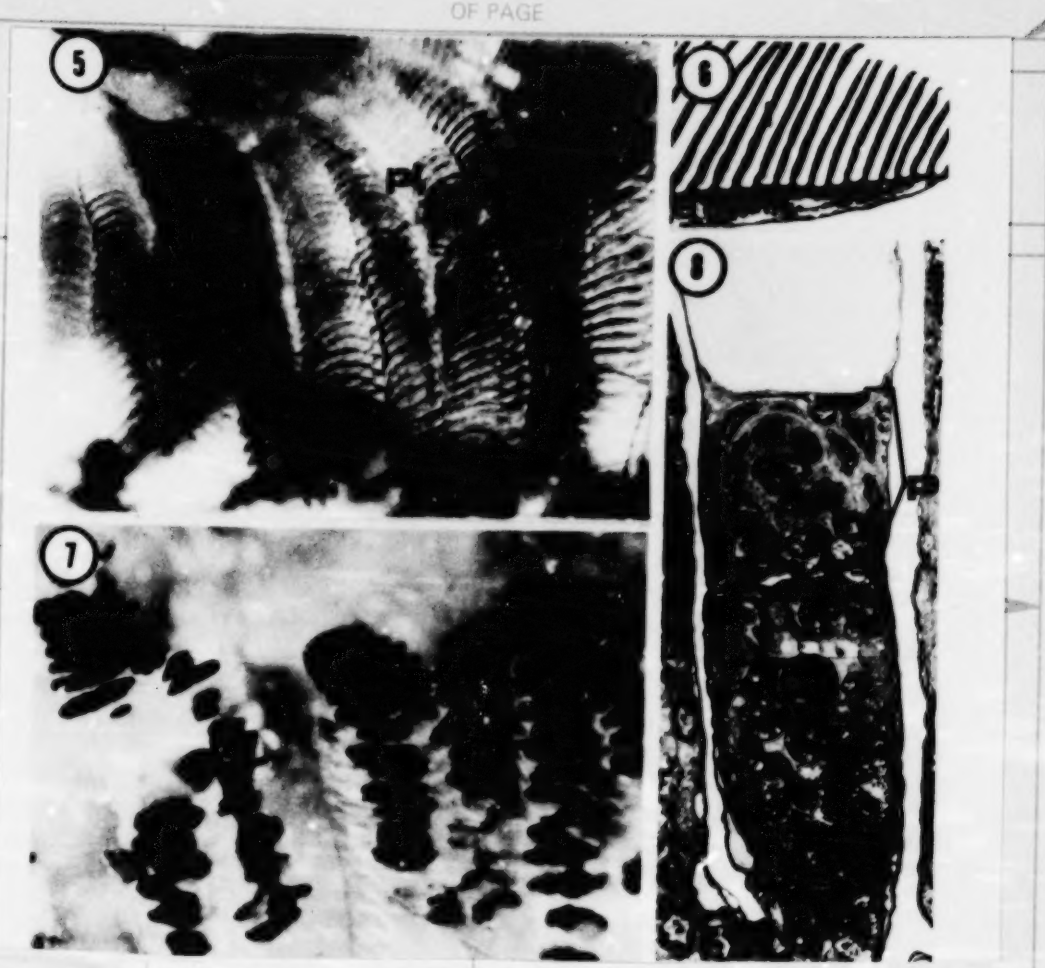

Fig. 5 Low power light micrograph of normal pleurobranchiae (pl) of Palaemonetes pugio. Note the translucent quality of the gill lamellae x 56.

Fig. 6 Sagittal section of a Toluidine-blue stained gill from grass shrimp showing normal appearance of axis (a) and attached lamellar plates (l) x 45.

Fig. 7 Light micrograph demonstrating the darkened (brownish-black) lamellar apices which characterize the gills from shrimp exposed to toxic levels of Busan-85 (14 days) and Aquatreat DNM-30 (3-4) days x 48.

Fig. 8 Sagittal section of Epon embedded black-gill lamelae characteristic of short-term exposure to Aquatreat DNM-30 or 14-day exposure to Busan-85. A dense hemocyte plug (P) occupies the lamellar apex and imparts a blackened appearance to the lamellae of living shrimp. Phagocytic hemocytes (H) occupy the basal lamella together with newly differentiated epithelial cells (E) which are in the process of forming a new cuticular layer (C) subjacent to the hemocyte plug x 360.

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Fig. 9 Sagittal section of a black gill from a newly molted grass shrimp exposed to Busan-85. The lamellae (1) are markedly truncated and possess extremely dense apical scabs (sc). x 300.

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Fig. 10 Light micrograph of sagittally sectioned black gills from shrimp exposed to Aquatreat DNM-30 for 3-4 days. Notice the electron dense scabs at the lamellar (l) apices. In contrast to the black gills from Susan-85 exposed shrimp as shown in previous figure, the blackened gills from Aquatreat shrimp exhibit a greater degree of cellular disorganization in the gill axis (Ax) and pronounced darkening of the hemolymph (He) x 360.

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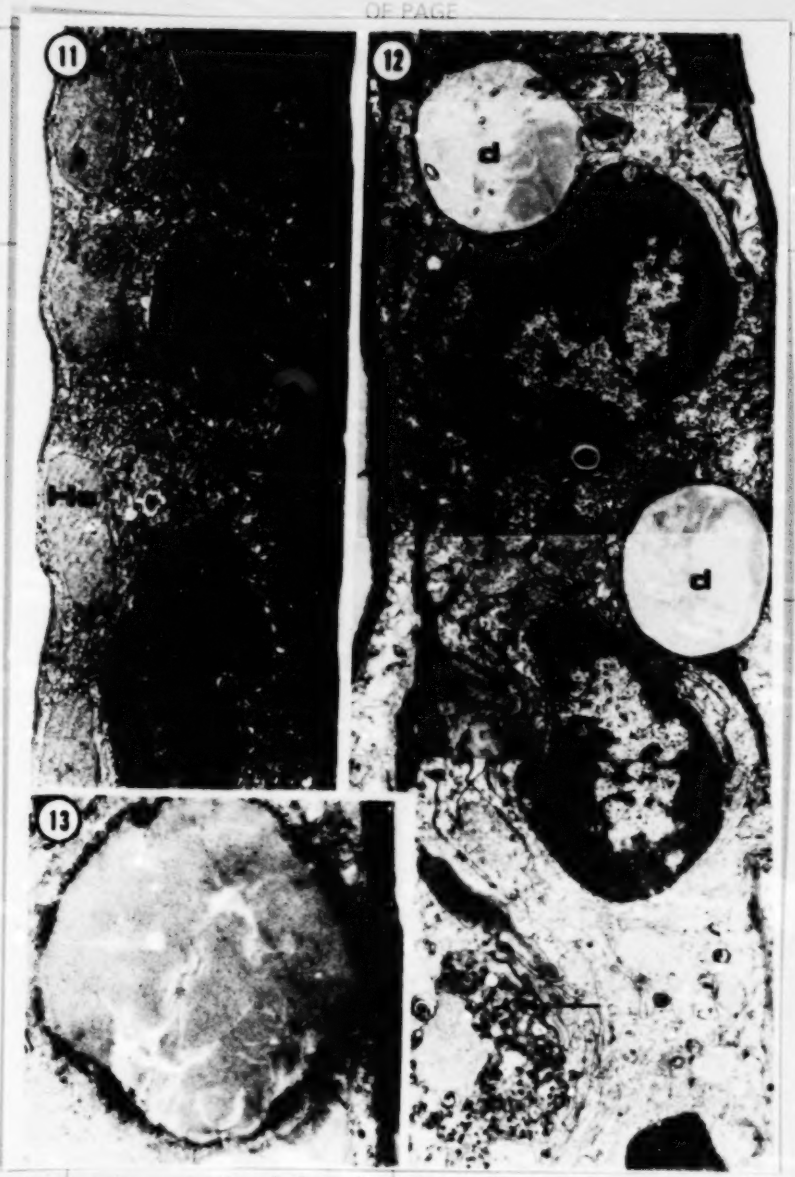

Fig. 11 Electron micrograph of lamella from shrimp exposed to Aquatreat DNM-30 for 3-4 days, demonstrating the abnormally electron-dense nature of the hemolymph (He) in the peripheral channels x 3,000.

Fig. 12 Lamella of shrimp exposed to Aquatreat DNM-30 (3-4 days) showing unusual lipid-like droplets (d) that appear to reside in hemolymph channels (ch). Note dense hemolymph (*) and dense mitochondrial inclusions (arrow) in degenerating epithelial cell x 4,500.

Fig. 13 Higher magnification electron micrograph of lipid-like droplet as seen in previous figure. Note the somewhat "fractured" appearance of the matrix and dense particulate material at periphery (arrow) x 9,000.

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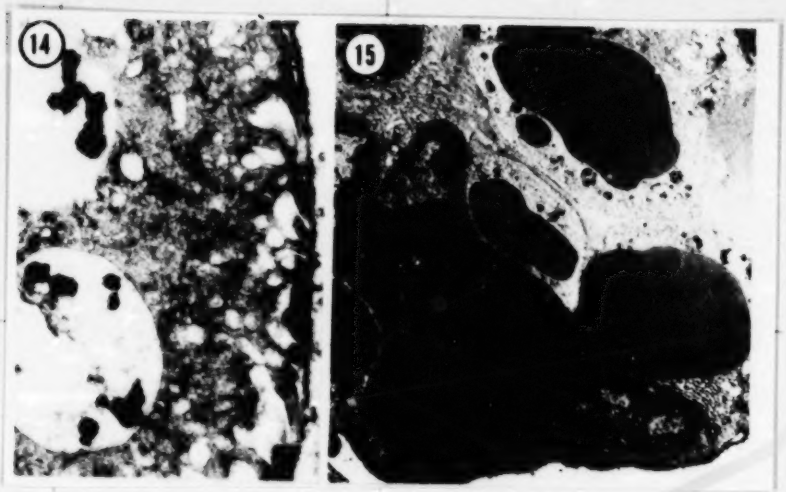

Fig. 14 Electron micrograph of abnormally electron-dense peripheral podocyte cytoplasm and abnormally structured pedicellar plasmalemma (p) from shrimp exposed to Aquatreat DNM-30 (3-4 days). x 25,000.

Fig. 15 Low power electron micrograph showing the abnormally dense appearance of the fluids contained in the central vacuoles (CV) of podocytes from shrimp exposed to Aquatreat DNM-30 (3-4 days). x 2,000.

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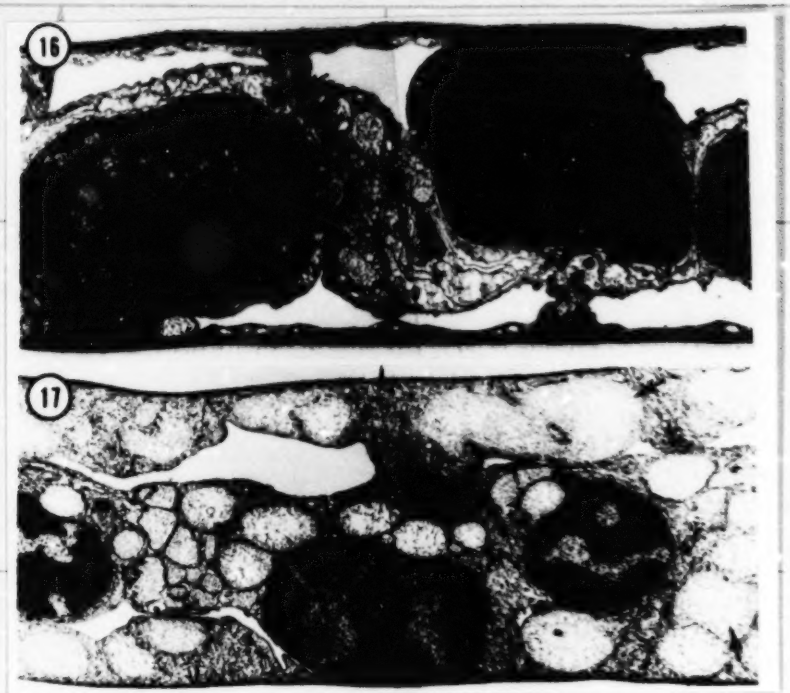

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Fig. 16 Sagittal section of lamella from shrimp exposed to Aquatreat DNM-30 for 28-35 days. Conspicuous electron-dense inclusions occur in the matrices of slightly swollen mitochondria (arrows). x 4,500.

Fig. 17 Lamella from shrimp exposed to Aquatreat DNM-30 (28-35 days) showing increased mitochondrial swelling (arrows) and a slight increase in lamellar thickness. x 3,000.

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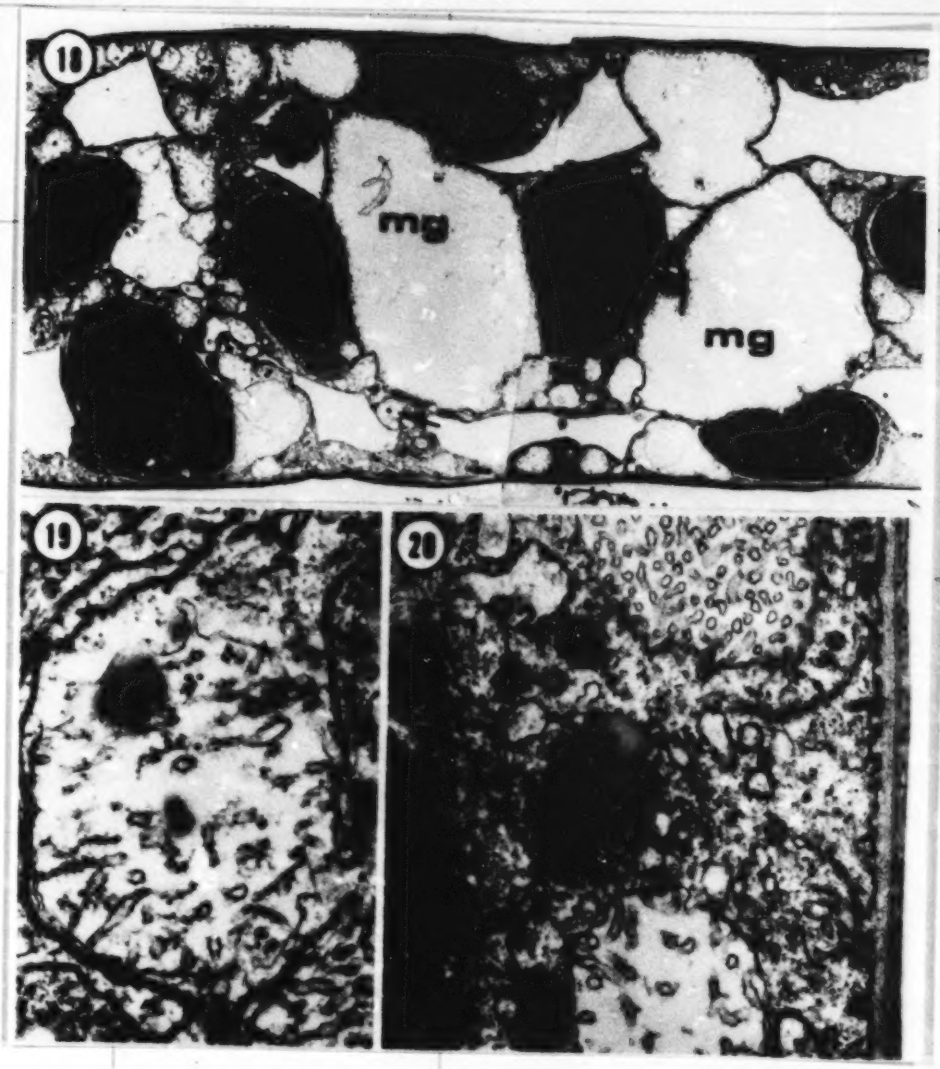

Fig. 18 A lamella characteristic of two shrimp exposed to Aquatreat DNM-30 for 28-35 days. Note the dense matrical inclusions (arrows) and megamitochondria (mg), as well as the grossly distended lamellar profile x 2,000.

Fig. 19 High magnification micrograph of amorphic lipid-like matrical inclusions (*) in lamellar mitochondria of shrimp exposed to Aquatreat DNM-30 (28-35 days) x 20,000.

Fig. 20 Congregations of dense particles (C_1) that appeared to condense (C_2) were common in the subcuticular lamellar epithelium of some shrimp exposed to Aquatreat DNM-30 for 28-35 days x 20,000.

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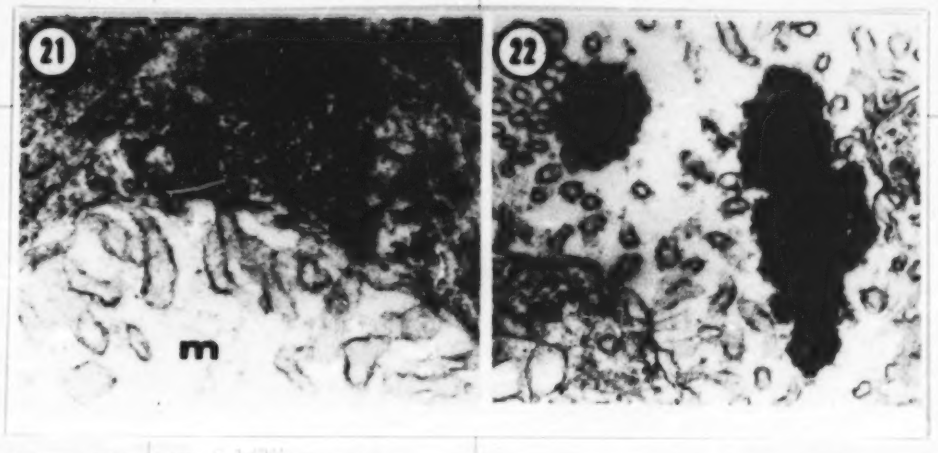

Fig. 21 A condensed grouping of particles (*) in apposition to the outer mitochondrial (m) membrane in the lamella of a shrimp exposed to Aquatreat DNM-30 for 28-35 days. x 45,000.

Fig. 22 High magnification of electron-dense matrical inclusions in lamellar mitochondria from shrimp exposed to Aquatreat DNM-30 for 28-35 days. x 30,000.

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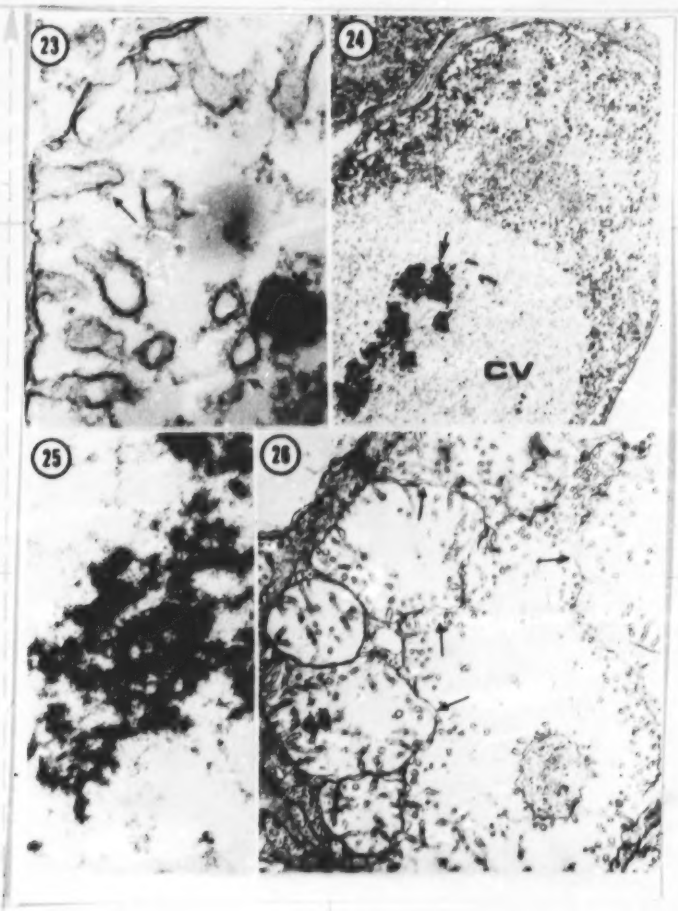

Fig. 23 High magnification of mitochondrial inclusion which seems to show the coalescence of a flocculent material (*) associated with the membranes of the cristae (arrows). Aquatreat DNM-30 (28-35 days) x 70,000.

Fig. 24 Dense material (arrow) in the central vacuole (CV) of an axis podocyte from shrimp exposed to Aquatreat DNM-30 for 28-35 days x 7,000.

Fig. 25 Higher magnification of substance shown in previous figure. Note the similarity between this material and the dense mitochondrial inclusions. Aquatreat DNM-30 (28-35 days) x 45,000.

Fig. 26 Micrography which shows that megamitochondria formation may proceed by the fusion of adjacent mitochondria. Arrows point to sites where only two membranes separate the matrices of adjacent mitochondria instead of the expected four membranes. From shrimp exposed to Aquatreat DNM-30 (28-35 days) x 15,000.